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14. ABSTRACT Our hypothesis is that reversible alterations in histones are a determining factor for low FANCD2 expression in ovarian surface epithelial (OSE) cells in women with a familial risk for ovarian cancer, and that cells with reduced FANCD2 levels are hypersensitive to the genotoxic effects of estrogen, therefore predisposing OSE to malignant transformation. During the study we have screened a large number of normal (no familial risk), high-risk (with familial history of this disease), and ovarian cancer cell lines, and determined levels of FANCD2 protein and mRNA. In the first set of experiments, aimed at determining whether histone modifications (i.e. acetylation and/or methylation) affect FANCD2 levels, we established that Trichostatin A (TSA;10nM for 24 hours) corrects FANCD2 levels but not consistently. In the second set of experiments, aimed at establishing whether the estrogen metabolite 4-OHE2 is genotoxic for cells with low FANCD2 levels we: 1) identified the minimal concentration of 4-OHE2 that is associated with DNA damage in human and murine OSE, and; 2) found that OSE cultures with low FANCD2 exhibit significantly increased DNA damage after exposure to 50 uM 4-OHE2, in comparison with OSE cultures with normal levels of FANCD2 protein expression.					
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## INTRODUCTION

Central to our application is our previous finding of decreased expression of FANCD2, a key protein in Fanconi (FA)/BRCA DNA repair pathway) in cultured ovarian surface epithelial (OSE) cells from women with a family history of ovarian cancer and women with ovarian cancer. We previously demonstrated that normalizing (increasing) FANCD2 expression in these cells improved their genetic stability, consistent with a causal relation between higher FANCD2, improved genetic integrity and decreased risk of ovarian cancer. The mechanism by which reduced FANCD2 may render OSE sensitive to neoplastic transformation is not known, but evidence suggested that such OSE cells may be sensitive to toxic effects of estrogen on genomic material and that loss of FANCD2 limits DNA repair potential in these cells. **The overall** objectives of the proposal were thus to delineate the molecular and genetic mechanisms underlying (1) reduced FANCD2 expression and (2) the etiology of cancer in FANCD2-deficient women. Our hypothesis was that reversible histone alterations (methylation and/or acetylation) were a determining factor for low FANCD2 expression in high risk OSE and that cells with reduced FANCD2 levels are hypersensitive to the genotoxic effects of estrogen, therefore predisposing OSE to malignant transformation. Two specific aims were pursued to test this.

### Specific Aims:

*I. Determine the epigenetic mechanisms of FANCD2 downregulation in the OSE of women at high risk for ovarian cancer, and to examine how normalizing FANCD2 expression might stabilize genome in high-risk women.*

*II. Evaluate estrogen-induced genotoxicity as an etiological agent in ovarian cancer in women with alterations in the FA/BRCA Pathway.*

## BODY – Summary

**Specific Aim 1:** Determine the epigenetic mechanism of reduced *FANCD2* expression in OSE cells from high-risk women, and whether treatment with histone deacetylase inhibitors (HDACi) improves survival of *FANCD2*-low cells and whether normalized *FANCD2* expression suppresses cross-linker-induced chromosomal breakage and cell toxicity.

1. We have amended our existing IRB approved protocols OHSU IRB 921 and OHSU 3485 to include Department of Defense sponsored research. Both IRB amended and approved protocols have subsequently been accepted by the DOD Research Protection Office approval.
2. Initially we cultured 100 human OSE samples from our Biorepository. Successful cultures were obtained from 78 human OSE cell lines. FANCD2 protein levels were determined using Western blot method in our laboratory, with Santa Cruz FANCD2 antibody. We have screened 17 normal OSE, 26 high-risk (HR) OSE and 35 ovarian cancer cell lines for FANCD2 protein and mRNA levels. The reduced levels of both FANCD2 protein and mRNA suggested epigenetic mechanism of FANCD2 regulation. We have previously shown that FANCD2 promoter is not methylated (1). However DNA histone modification may also regulate gene expression (2). Therefore, cells with reduced FANCD2 levels were treated with trichostatin-A (TSA) at 10 nM concentration, 5-aza-2'-deoxycytidine (5-aza) 0.5 uM and combination of TSA and 5-aza and several time points. The changes in FANCD2 protein levels were evaluated by densitometry. The pattern of response to TSA and 5-aza was variable (Figure 1) and it remained so when larger number of samples (18) was tested for TSA response.

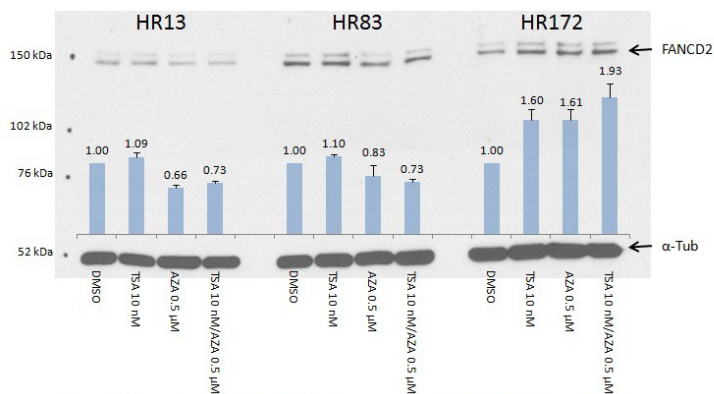
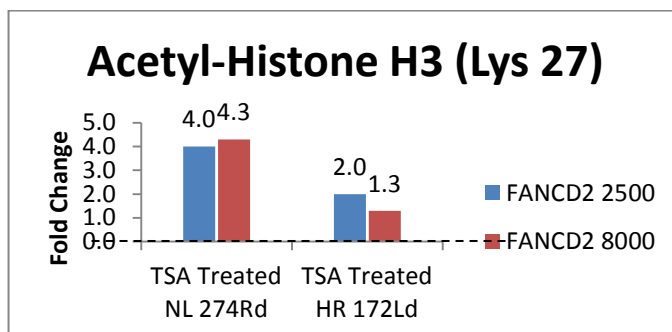


Figure 1. Three primary high risk cell lines that were treated with 10nM Trichostatin A (TSA) and 0.5 $\mu$ M 5-Aza-2'-deoxycytidine (AZA). Top band located at approximately 155 kDa is the FANCD2 protein. Lower band at approximately 50 kDa is loading control alpha-tubulin. Graph represents adjusted density of band as compared to DMSO using Image J by NIH.

We then selected appropriate cases after treatment with 300nM Trichostatin A (TSA) for 24 hours. For chromatin immunoprecipitation (ChIP) cells were crosslinked with 1% formaldehyde for 10 minutes at room temperature and washed with PBS twice. Chromatin was sheared to 200-1000 kb using a Branson Digital Sonifier 450, amplitude of 35% with 15 pulses of 10 seconds. Shearing was verified by 1% agarose gel. Immunoprecipitation of cross linked protein/DNA was done using reagents from Magna ChIP HiSens kit from Millipore. Chromatin was precleared with Magna ChIP Protein A/G Magnetic Beads and then incubated overnight at 4°C with 5 ug ChIP validated antibodies from Millipore, CHIPAb+ Acetyl-Histone H3 (Lys27) mouse monoclonal Antibody/Primer set with Normal Mouse IgG (Cat# 17-683). Magnetic beads were then added and incubated overnight at 4°C. Samples were washed and eluted following Magna ChIP HiSens protocol. Reverse crosslinking was done in ChIP Elution Buffer for two hours at 65°C with 1 volume of Proteinase K.

Real-time quantitative PCR of 2  $\mu$ l enriched ChIP DNA was performed using ABI 7500 Fast and Power SYBR Green PCR Master Mix. Reactions were 20  $\mu$ l in triplicate with 50 cycles of 94°C 20 seconds and 60°C for 1 minute per antibody manufacturer's protocol. Data analysis was done using  $\Delta\Delta$ Ct method. Primer set was FANCD2 2500 base pairs from transcription start site (FANCD2 2500): FOR: CCA CCT CAT AGA GTC CAG CCC ACT, REV: GGA AGC CCC ACT TCA TAG CTC CTT; and FANCD2 8000 base pairs from transcription start site (FANCD2 8000): FOR: CAC CTT AAC TGA GAT GCC CGG AAC, REV: ATG AAA TGA GCT GGG GTT TTG CAT. Neither Acetyl-Histone H3 (Lys27)(Figure2) nor Lys9/15 showed significant fold change between TSA treated and non treated high risk or normal OSE. Therefore for the primary OSE cell lines tested and antibodies against lysine residues tested our hypothesis has been disproved.

Figure 2. Fold change of TSA treated NL 274Rd and HR 172Ld at primer sites 2500 base pairs from TSS and 8000 base pairs from transcription site. Fold change of 1 equals no change as compared to control.



**Specific aim 2: We aim to evaluate estrogen-induced genotoxicity as an etiological agent in ovarian cancer in women and mice with alterations in *FANCD2* expression and to determine if reduced *FANCD2* expression renders OSE cells sensitive to estrogen-induced chromosomal instability.**

Comet assay was used to assess genotoxicity of the estrogen metabolite 4-hydroxyestradiol (4-OHE2) in vitro, in 20 human OSE cell lines with various FANCD2 protein levels and 2 mouse cell lines (wild type and Fancd2 <sup>-/-</sup> mouse). Collected cells were brought to 1.5x10<sup>5</sup> cell/mL with PBS. Comet assay was performed using Trevigen Comet Assay reagents for Single Cell gel Electrophoresis. Cells were suspended in low melting point agarose, plated on slides and allowed to harden for 10 minutes at +4° C. Slides were then washed, incubated in 70% ethanol and allowed to dry overnight at room temperature. Samples were stained with SYBR Green and viewed using fluorescent microscope. Images were analyzed by scoring 100 cells per condition and determining percent DNA in tail using CometScore (TriTek Corp, Sumerduck, VA, USA). Primary OSE cells were cultured in Phenol Red-Free DMEM/RPMI (GIBCO, Life Technologies) with 10% Charcoal/Dextran treated FBS (ThermoScientific) in 6 well plates with 2 x 10<sup>6</sup> cells per well. Untreated cells were included as negative controls. Cells treated with 50 uM Camptothecin were used as positive controls. Experimental cells, to test the effects of the estrogen metabolite, were exposed for 2 hours with 50 uM and 75 uM 4-OHE2 at 37 °C and then harvested by trypsinization. The data was analyzed using percent DNA in tail (CometScore).

A non-parametric test was used to assess the differences in DNA damage between 11 cell lines with reduced FANCD2 levels and 8 FANCD2 positive cell lines. Only 50 uM 4-OHE2 showed significant differences in this assay (Table 1).

**Table 1.**

	Low (n=11)	Positive (n=8)	T test P-value	P-value *
Control, Mean(SD)	4.43 (0.96)	4.66 (0.15)	0.4880	0.1731
Camp, Mean(SD)	80.49 (6.93)	75.27 (9.37)	0.1866	0.2006
50uM, Mean(SD)	7.80 (2.68)	13.53 (7.11)	0.0445	0.0093
70uM, Mean(SD)	18.60 (12.22)	33.33 (16.03)	0.0358	0.1074

\* Wilcoxon- Mann-Whitney test (Nonparametric test)

The DNA damage response to 4-OHE2 in low FANCD2 expressing cells was not as uniformly increased as expected. One explanation may be in that the Comet Assay detects DNA damage in the form of DNA single strand breaks but also cross-links which move slower within Comet assay and are therefore not as readily detected as single strands DNA products(3, 4); therefore, an alternative technique to detect DNA damage - cytogenetic analysis to specifically detect chromosomal breaks and radials was used. The results however showed increased levels of radials and chromosome breaks after 4hr estradiol exposure in both normal and high-risk OSE. The difference in level of DNA damage, as measure by cytogenetic parameters was not statistically significant between the control and high-risk OSE.

Similarly, the two murine cell lines showed increased DNA tails with exposure to 25 microMol 4OHE2 however the additional experiments revealed that results were not reproducible in the larger cohort e.g, estradiol treatment had no detectable effect on FANCD2 expression in any of the lines either globally nor when FANCD2 nuclear and cytoplasmic fractions were treated separately.

In the course of the work we were able to establish and detect cytoplasmic and nuclear components of FANCD2 protein. Moreover we have identified cell lines with predominately cytoplasmic FANCD2. Furthermore we have detected an association between the presence of cytoplasmic FANCD2 and trend towards better survival of women whose tumors expressed mostly cytoplasmic fraction of FANCD2.

### **Key Research Accomplishments:**

1. We identified the minimal concentration of 4-OHE2 that is associated with DNA damage in human and murine OSE.
2. FANCD2 low OSEs exhibit significantly increased DNA damage after exposure to 50 uM 4-OHE2 in comparison with OSE with normal FANCD2 protein expression.
3. We identified that TSA corrects the FANCD2 level inconsistently in some high-risk OSE at concentration of 10 nM after 24 hours exposure. We could not identify any correlation between these results and modification of H3 lysine residues by ChIP analysis.
4. We have identified, for the first time, in OSE, the presence of cytoplasmic FANCD2. This has potential clinical implications as the presence of cytoplasmic FANCD2 seems to correlate with better survival in women with ovarian cancer.

### **Reportable Outcomes:**

#### Publications:

Pejovic T, Mc Winney S, Hays LE: Beyond Heredity Mutations: Alterations in the Fanconi Anemia/BRCA Pathway and its Role in Ovarian Tumorigenesis. Journal of Experimental Obstetrics and Gynecology, 2014, accepted.

Wright JW, Engel S, Kellar M, Bean Y, Hays L, Mhawech-Fauceglia P, Lim YJ, Pejovic T: Subcellular localization of FANCD2 is associated with survival in serous carcinoma of the ovary. Manuscript submitted. 2014

Meeting abstracts: Kellar M, Bean Y, Wright J, Pejovic T: Estrogen does not modulate FANCD2 in high-risk ovarian surface epithelial cells. Western Association of Gynecologic Oncologist, Annual meeting June 2014, poster.

Cell lines: Developed cytoplasmic and nuclear FANCD2 cell lines.

Tissue and serum

repositories: None Informatics and

databases: None Animal models:  
None

Funding applied for based on this award:

- 1.Marsha Rivkin Ovarian cancer Research Award (1 –year award)
- 2.DOD Translational Leverage Award 2014, submitted based in part on the results related to FANCD2 expression.

Employment or research opportunities applied for or received based on this award: 1.Melissa Kellar, B.Sci. her funding was supported by this DOD award leading to now her retention within the institution.

**Conclusion:** Our results support the notion that (1) TSA may correct FANCD2 expression in ovarian epithelial cells characterized by low FANCD2 expression but this increase does not correlate with modification of Histone 3 lysine residuals, (2) estrogen byproduct 4-OHE2 induces cDNA damage in human and mouse cells with low FANCD2 and (3) cytoplasmic fraction of FANCD2 is a dominant fraction in significant portion of high-risk and cancerous OSE.

#### **References:**

Pejovic T, Yates JE, Liu HY et al (2006): Cytogenetic instability in ovarian epithelial cells from women at risk of ovarian cancer. *Cancer Res* 66:9017-9025.

Jones PA, Baylin SB (2002). The fundamental role of epigenetic events in cancer. *Nat Rev Genet*, 3: 415-428.

Tice RR, Agurell E, Anderson D et al (2000): Single cell gel/comet assay: Guidelines in vitro and in vivo genetic toxicology testing. *Environ Mol Mutagen* 35:206-221.

Wu JH, Jones NJ (2012): Assessment of DNA interstrand crosslinks using the modified alkaline comet assay. *Methods Mol Biol* 817:165-81.

#### **Appendices:**

Manuscripts submitted.  
Poster WAGO 2014.



# Beyond Heredity Mutations: Alterations in the Fanconi Anemia/BRCA Pathway and its Role in Ovarian Tumorigenesis

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## Abstract:

The Fanconi anemia (FA)/BRCA pathway consists of fifteen proteins that mediate DNA homologous recombination and promote chromosomal instability. Here we review the evidence that genetic and epigenetic alterations in *BRCA2* (*FANCD1*), *BRIP1* (*FANCJ*), *FANCD2*, *FANCF*, *PALB2* (*FANCN*) and *RAD51C* (*FANCO*), render ovarian cells susceptible to malignant transformation. In addition, we discuss the paradoxical findings that *BRCA2* and *FANCD2* are upregulated in subsets of ovarian cancer patients and how these findings suggest potential tailored therapies.

## Introduction:

A growing body of evidence demonstrates that proteins in the Fanconi anemia/BRCA (FA/BRCA) DNA repair pathway play pivotal roles in ovarian cancer suppression. Fanconi anemia is an inherited bone marrow failure syndrome that is characterized by chromosomal instability and hypersensitivity to DNA cross-linking agents, including cisplatin. In fact, increased chromosomal breakage in response to DNA cross-linkers (mitomycin C and diepoxybutane) is the accepted diagnostic test for FA. In addition to anemia, FA patients are highly predisposed to leukemia, head and neck squamous cell carcinoma, and gynecological cancers (cervical and vulvar)<sup>1</sup>.

FA is caused by homozygous or X chromosome-linked deletion of one of 15 different genes, eight of whose gene products (*FANCA*, *FANCB*, *FANCC*, *FANCE*, *FANCF*, *FANCG*, *FANCL*, and *FANCM*) form a nuclear core complex that facilitates ubiquitination of *FANCD2* and *FANCD1*. The ubiquitinated *FANCD2*/*FANCD1* heterodimer then functionally interacts with down-stream FA proteins (*FANCD1*/*BRCA2*, *FANCJ*/*BRIP1*, *FANCN*/*PALB2*, *FANCO*/*RAD51C*, and *FANCP*/*SLX4*) to mediate DNA damage responses. The role of these proteins in promotion of chromosomal stability by mediating repair of DNA cross-links and double-strand breaks has been well established. In fact, FA proteins functionally interact with many members of the DNA homologous recombination (HR) pathway, including *BRCA1*. Although HR proteins and others interact with FA proteins, only genes that have been demonstrated to cause Fanconi anemia are designated as FANC genes and are the focus of this review (Table 1).

Although FA proteins play an essential role in suppressing chromosomal instability, there is substantial evidence that these proteins are multi-functional. Specifically, FA proteins suppress hyper-responsiveness to apoptotic cues<sup>2-4</sup>, over-production of inflammatory cytokines<sup>3,5-7</sup> and overproduction of reactive oxygen species.<sup>8,9</sup> The potential contribution of these other functions of FA proteins to ovarian malignant transformation remains unclear. However, a recent report demonstrated that loss of cytoplasmic FANCD2 staining in breast cancers strongly correlated with poor prognosis, suggesting an important non-nuclear function of FANCD2 in breast cancer suppression.<sup>10</sup>

In 2002, the *FANCD1* gene was identified to be *BRCA2*, emphasizing the importance of this pathway in suppression of ovarian tumorigenesis. However, there is no increased incidence of ovarian cancer in (1) FA patients which may in part be due to early mortality (median age of survival is xxx) from other pathologies (bone marrow failure or squamous cell malignancies) or (2) in parents of FA patients. This may be accounted for by the fact that the vast majority of FA is caused by mutations in three FA genes (*FANCA*, *FANCC*, and *FANCG*) that have not been implicated in ovarian cancer. Although, one case of breast cancer in an *FANCA* patient and a small increased susceptibility of *FANCC* carriers to breast cancer has been reported. Subsequent work by different groups have identified, however, that at least three of the less common FA genes are involved in hereditary ovarian cancer susceptibility (*FANCI/BRIP1*, *FANCN/PALB2*, and *FANCO/RAD51C*). Other evidence suggests that epigenetic changes in and altered expression of FA genes contributes to both sporadic and hereditary ovarian cancer. This review will detail the evidence supporting the important role of the FA/BRCA pathway members in both suppression and promotion of ovarian cancer and how these FA/BRCA alterations may direct tailored treatments.

#### Hereditary Mutations in Ovarian Cancer:

It has been reported that approximately 10% of ovarian cancers are believed to be hereditary and the vast majority of these cases have been attributed to mutations in the *BRCA1* and *BRCA2* genes. Bi-allelic inactivation of *BRCA2* (*FANCD1*) causes Fanconi anemia, with a particularly severe phenotype that includes brain tumors. Recently other hereditary mutations have been found that confer ovarian cancer susceptibility and many of which are in the FA pathway, including *FANCI* (*BRIP1/BACH1*), *FANCN* (*PALB2*), and *FANCO* (*RAD51C*). Although the relative proportion of these mutations in hereditary ovarian cancer and the total number of women with hereditary ovarian cancer was believed to be small, Walsh recently reported that the overall percentage of women with hereditary mutations was higher than thought (24%) and many women with hereditary mutations present without a family history (>30%).<sup>11</sup> Furthermore, they detected by parallel sequencing in a method they named 'BROCA' that 6% of all ovarian cancers tested had mutations in other genes, with 2% having mutations in non-*BRCA2* FA genes (*FANCI*, *FANCN*, and *FANCO*). The contribution of FA genes to ovarian tumorigenesis may in fact be higher as (1)

BROCA testing does not include other FA genes (although mutations in other FA genes may be exceedingly small as FA parents are not pre-disposed to breast or ovarian cancer, as described above) and (2) as will be discussed below non-genetic alterations in the FA pathway may play an important role in ovarian tumorigenesis. As the evidence and connection of the FA proteins to hereditary ovarian cancer was comprehensively reviewed by Pennington and Swisher<sup>12</sup> in this review will focus on evidence for non-genetic alterations in the FA pathway that contribute to both sporadic and hereditary ovarian cancer.

### Epigenetic Regulation of FA Pathway Members:

#### *FANCF Methylation:*

A possible role of altered FANCF expression in ovarian cancer, interestingly the only core complex FA protein so far implicated in the etiology of ovarian cancer, was first reported in 2003.<sup>13</sup> Taniguchi et al demonstrated that two ovarian cancer cell lines were deficient for mono-ubiquitinated FANCD2 due to methylation-mediated loss of *FANCF* expression. Furthermore they found that 21% (4/19) of primary ovarian tumors from patients with no history of breast or ovarian cancer displayed *FANCF* methylation, while corresponding peripheral blood samples did not. Subsequent reports by other groups have varied in the relative proportion of *FANCF* methylation positive ovarian cancer samples. Specifically, two groups found, respectively, that 27.8% (5/18)<sup>14</sup> and 13.2% (x/53)<sup>15</sup> of primary ovarian tumors possessed *FANCF* methylation, while in contrast two others found either no (0/106)<sup>16</sup> or low (2.2%; 3/143)<sup>17</sup> levels of methylation. Differences may be due to stage, histological subtype, or treatment history as exemplified by Swisher et al that showed that a small but detectable fraction (3%; 3/93) of primary tumors (pre-treatment) displayed methylation (3/93), but no tumors from post-treatment or recurrent patients displayed methylation.<sup>17</sup> Alternatively, differences may be due to ethnic compositions of tumor samples from where they were collected, i.e. United States<sup>13,17</sup>, China<sup>14</sup>, and United Kingdom<sup>15,16</sup>. Regardless, the preponderance of evidence suggests that a small, but not insignificant proportion, of sporadic ovarian cancers are positive for *FANCF* methylation, which may have important considerations for treatment as discussed later in the review.

Mice deficient for the murine homolog of FANCF (*Fancc*) display small ovaries with reduced follicles and spontaneously develop ovarian tumors, demonstrating a critical function for FANCF in ovarian function.<sup>18</sup> The tumors from these mice are granulosa cell and luteomas, which correlates with another study that shows that 24% (6/25) of human ovarian granulosa cell tumors are methylated at *FANCF*.<sup>19</sup> Therefore, aberrant FANCF function may play a critical role in both epithelial and non-epithelial ovarian cancers.

#### *Methylation of Other FA Genes (FANCN/PALB2 and RAD51C):*

A few reports have suggested that methylation of other FA genes may contribute to ovarian tumorigenesis. Potapova et al found that 7.5% (4/53) of sporadic ovarian tumors and 0% (0/9) of inherited tumors contained methylation at the *FANCN/PALB2* gene.<sup>20</sup> Although the numbers are small, this report suggests that analogous to *BRCA1* and *BRCA2*, alterations in expression of *FANCN/PALB2* may contribute to both inherited and sporadic ovarian cancer. In addition, one recent report detected *RAD51C* promoter methylation in 2.5% (1/39) of hereditary ovarian cancer patients without *BRCA1/2* mutations. The numbers of tumors tested in both of these studies were small, demonstrating the need for further studies for confirmation. It should be noted that methylation of individual FA genes in ovarian cancer may be small, but together may add up to a significant proportion and as alterations in these different genes renders cells susceptible to the same molecular agents (i.e. platinum agents and PARP inhibitors), implications for putative treatments would be the same.

The use of murine models to clarify the roles of *Fancn* and *Fanco* in ovarian tumorigenesis are limited by the fact that whole animal knockouts of these genes are embryonic lethal, analogous to *Brca1* and *Brca2*.

#### Other Alterations in the FA Pathway

In addition to epigenetic changes, several groups have reported altered FA gene expression in ovarian cancer samples, although the putative molecular and genetic mechanisms underlying aberrant expression have not been delineated.

##### *FANCD2*:

In work done by our group, expression of *FANCD2* protein and mRNA was found to be down-regulated in 60% (3/5) of pathologically normal ovaries from women with a family history of ovarian cancer (but no *BRCA1/2* mutations) and 12.5% (1/8) of women with ovarian cancer.<sup>21</sup> *FANCD2* reduced expression was restricted to the ovaries as normal *FANCD2* expression was detected in peripheral blood from these same patients. In the ovaries, no DNA mutations were detected in the *FANCD2* gene or its promoter nor was aberrant methylation detected in *FANCD2* or ten other FA genes screened. Although the numbers were small in the original study, we have found significant *FANCD2* down-regulation in an expanded study (unpublished data). To further support a role for *FANCD2* in ovarian tumor suppression, another group found that *FANCD2* mRNA expression was low in borderline and stage III ovarian tumor samples.<sup>22</sup>

The mechanism underlying repressed *FANCD2* expression remains enigmatic, but several possibilities include aberrant chromatin and miRNA dysregulation. To support the latter, one group identified a novel genetic variant in the *miR-191* gene in an ovarian cancer family that increased its own expression. One of the putative targets of this miRNA is *FANCD2* and ex vivo experiments demonstrated *miR-191* overexpression suppresses *FANCD2* expression by approximately 40%. Further experiments are needed to identify the mechanisms involved in

FANCD2 repression, but may be of particular interest as they could potentially function as targets for early therapeutic intervention to restore FANCD2 expression and prevent malignant transformation in high-risk women.

To support an important role for FANCD2 in ovarian development and malignant transformation, mice deficient for *Fancd2* display underdeveloped ovarian follicles and the majority of mice develop epithelial ovarian tumors after 15 months of age. Murine models for *Fancd2* and the other FA genes described above allow delineation of the contribution of these genes to the etiology of FA-related ovarian cancer and furthermore function as pre-clinical models for potential tailored therapies.

#### Loss of FA Pathway and Treatment

PARP inhibitors were proposed to be efficacious in cells deficient for BRCA1 and BRCA2 due to synthetic lethality and have shown promise in treatment of BRCA1/2-deficient breast cancer. The efficacy of these drugs in BRCA1/2-deficient ovarian cancers is still under investigation. Analogous to BRCA1/2-deficient cells, cells with deficiencies in FA genes (*FANCA* and *FANCD2*) are hypersensitive to PARP inhibitors. Therefore, cells with alterations in FA genes or FA gene expression, identified by BROCA testing or other means, may be sensitive to the same strategies proposed for BRCA1/2 positive tumors.

As loss of FA/BRCA pathway most likely promotes malignant transformation as an early event through genomic instability, strategies destined to either restore FA/BRCA expression or eliminate cells deficient for FA/BRCA genes before tumor formation present a tantalizing possibility. For example, as discussed above, FANCD2 expression was found to be low in a majority of non-cancerous ovaries (but not peripheral blood) from high-risk women. If the mechanism of FANCD2 repression could be identified, therapies that would restore FANCD2 repression may be developed to potentially inhibit malignant transformation in these women.

#### Paradoxical Upregulation of FA/BRCA Pathway in Sporadic Ovarian Cancer:

It is clear from the evidence cited above that many of the FA/BRCA proteins function as tumor suppressors, as reduced expression of these genes either through DNA mutations, promoter methylation, or other unknown molecular alterations correlate with ovarian cancer susceptibility and furthermore murine models of many of the genes spontaneously develop ovarian cancer. However, paradoxically, overexpression of these genes may also promote ovarian tumorigenesis as upregulation of these genes has been detected in ovarian tumors. Specifically, *BRCA2* mRNA was found to be over-expressed in tumor tissue vs non-tumor tissue in sporadic ovarian cancer<sup>23</sup> and high expression of FANCD2 protein correlated with women with poor prognosis in our study.<sup>24</sup>

The contribution of FA gene overexpression to ovarian cancer is unknown, however one likely possibility is that FA genes are upregulated after chemotherapy as a mechanism of carboplatin resistance. To support this, FANCD2 overexpression was highest in women with recurrent disease of less than one year. Regardless of the mechanism, upregulation of these genes and their protein products would most likely render these cancers resistant to both DNA cross-linkers and strategies that are dependent on loss of the FA/BRCA pathway (i.e. PARP inhibitors). It has been proposed by several groups that targeted inactivation of the FA/BRCA pathway with chemicals such as curcumin may be an effective strategy for sensitizing tumors to crosslinkers. These tumors might be ideal candidates for these treatments.

### Bioinformatics and Tailored Therapies

As discussed above, alterations in the FA/BRCA pathway may make ovarian cancer cells more sensitive to specific therapies. Therefore, identification of alterations in these pathways in both hereditary and sporadic tumors is an important goal. Currently, this can be accomplished by *BRCA1/2* sequencing at Myriad Genetics, BROCA sequencing at the University of Washington, and different other gene panels by several companies. However, molecular and epigenetic alterations that render ovarian cancer cells sensitive or resistant to FA/BRCA specific treatments would most likely not be detected by traditional DNA sequencing. We have performed a comprehensive analysis of publically available TCGA ovarian cancer data set for the six FA genes of interest (Figures 1-3) including DNA mutation and mRNA expression status and most frequently altered gene network. This analysis revealed the alteration in over 35% of ovarian cancer cases. Therefore, this and other bioinformatical approaches including gene expression arrays and miRNA expression arrays will be successful in identifying other women that would be responsive to these therapies.

Figure 1-3: FA genes/proteins and in TCGA data set.

Table 1: FA Genes and Relationship to Ovarian Cancer

FA Gene	Other Names	Function*	Association with Ovarian Cancer
<i>FANCA</i>	–	Core	–
<i>FANCB</i>	–	Core	–
<i>FANCC</i>	–	Core	–
<i>FANCD1</i>	<i>BRCA2</i>	Downstream	Hereditary mutations and epigenetic misregulation
<i>FANCD2</i>	–	Downstream	Decreased expression in HR women and overexpression in sporadic cases with poor prognosis
<i>FANCE</i>	–	Core	–

<i>FANCF</i>	–	Core	Hypermethylation in sporadic OvCa
<i>FANCG</i>	–	Core	–
<i>FANCI</i>	–	Downstream	–
<i>FANCI</i>	<i>BRIP1/BACH1</i>	Downstream	Hereditary mutations
<i>FANCL</i>	–	Core	–
<i>FANCM</i>	–	Core	–
<i>FANCN</i>	<i>PALB2</i>	Downstream	Hereditary mutations and hypermethylation (?)
<i>FANCO</i>	<i>RAD51C</i>	Downstream	Hereditary mutations
<i>FANCP</i>	<i>SLX4</i>	Downstream	–

\*For simplicity, function is defined here as member of the nuclear core complex (core) or functioning downstream of the core complex (downstream).

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# SUBCELLULAR LOCALIZATION OF FANCD2 IS ASSOCIATED WITH SURVIVAL IN SEROUS CARCINOMA OF THE OVARY

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Running Title: FANCD2 LOCALIZATION AND OVARIAN CANCER SURVIVAL

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## **Abstract**

**Summary:** Ovarian cancer risk is mitigated by a number of factors, including ovarian function, environmental influences, and genetic and epigenetic effects. Here, we examine an additional factor: the role of localization of FANCD2, a DNA repair protein as a contributor to ovarian cancer risk and patient survival. In the current study, we examined localization of FANCD2 into nuclear versus cytoplasmic compartments, and found that increased cytoplasmic FANCD2 (cFANCD2) correlated significantly with increased patient survival.

**Methods:** Tissue samples collected from consenting patients with or without ovarian cancer were evaluated by Tissue Microarray to determine whether cFANCD2 levels correlated with survival. Expression and localization was examined *in vitro* in normal and ovarian cancer cells.

**Results:** Patients with higher levels of cFANCD2 experienced significantly longer median survival time (50 months), versus patients with decreased cFANCD2 (38 months;  $p < 0.04$ ). Furthermore, it was observed that normal ovarian cells consistently expressed higher levels of nuclear and cFANCD2 than did most cancer cells.

**Conclusion:** Decreased cFANCD2 in cancer cells may indicate a susceptibility to transformation by their progenitors; meanwhile, elevated cFANCD2 suggests this susceptibility may be leveraged for improved patient outcome. cFANCD2 may be both an indicator of cancer risk, and prognosis in cancer patients.

## Introduction

Epithelial ovarian cancer has been a recalcitrant disease to address: the annual incidence of diagnosis and mortality rates have remained marginally improved for decades, with approximately 21,000 diagnoses and 15,500 deaths annually in the United States since the 1970's (Bast, 2011; Siegel et al., 2013). The frontline treatment strategies include surgical cytoreduction to decrease tumor mass, followed by chemotherapy to reduce the rate of additional tumor growth (Baldwin 2012). The initial chemotherapeutic regimens are platinum-based, however tumor cells almost inevitably acquire resistance to these compounds, tumor growth resumes, and patients succumb to the disease (Kelland, 2005).

Because of the nature of ovarian cancer, with symptoms that are difficult to detect at early stages, and with later stages presenting vague or nonspecific symptoms (Bankhead et al., 2008), it must be accepted that improvements in detection and understanding of the molecular basis of this disease will provide the most beneficial clinical prospects to increase patient survival and decrease disease incidence. In particular, identification of specific risk factors and specific molecular defects may allow the development of patient-specific treatments for optimal survival.

The significance of endogenous DNA repair pathways in ovarian cancer has been well established (Pejovic et al., 2006; Wysham et al., 2012). On one hand, inherited or acquired defects in DNA repair can be predictive of cancer risk (Pejovic et al., 2006). On the other hand, detecting defects in DNA repair has prognostic value: tumors that have a diminished capacity for DNA repair may be more susceptible to platinum-based therapies (Wysham et al., 2012). The ability to modulate DNA repair pathways may

therefore be of immense clinical value in cancer prevention via restoration of these pathways prior to transformation, and in patient survival, via disruption of these pathways in tumors.

The Fanconi anemia (FA) pathway of DNA repair may be a promising target for both ovarian cancer prevention and treatment. The FA pathway is composed of a large complex of proteins, involving 15 distinct genes. This complex overlaps with the BRCA1/2 pathway, and thus forms an integrated FA/BRCA pathway. Multiple members of this DNA repair pathway have been implicated in ovarian cancer risk, either due to mutation (BRCA1/2) (Meric-Bernstam et al., 2013; Paul and Paul, 2014) or reduced expression (FANCD2, FANCF, FANCN, FANCO) in the absence of genetic mutation (Pennington and Swisher, 2012).

Here, we focus on FANCD2. It has been demonstrated that reduced expression of FANCD2 correlates with a familial risk for ovarian cancer (Pejovic et al., 2006). In addition, in the current study we suggest potential defects in trafficking of FANCD2 between the nucleus, where it is functionally active in DNA repair, and the cytoplasm, where it has no established function (Bagby, 2003). Improper subcellular localization of proteins has been implicated in risk and prognosis in a variety of cancers (Singh et al., 1998; Henderson, 2012; Mesplede et al., 2012; Song et al., 2013), including benign breast tissue samples that show both cytoplasmic and nuclear distribution of FANCD2 (Rudland, 2010). In Tissue Microarray Analysis of 181 ovarian cancer samples, we report a significant ( $p < 0.04$ ) increase in survival time for patients with elevated cytoplasmic FANCD2 (cFANCD2). Furthermore, we observed significant ( $p < 0.02$ ) decreases in cFANCD2 in cultured ovarian cancer cells versus ovarian cells from

normal patients. This suggests that loss of the nuclear component, where DNA repair occurs, may improve the efficacy of platinum-based treatment to lethally compromise the genetic integrity of tumor cells.

While the mechanism(s) of FANCD2 subcellular localization are currently unknown, the association between elevated cFANCD2 and cancer prognosis suggests a novel avenue for cancer treatment, through amplifying cFANCD2 in tumor cells. Alternatively, modulation of cFANCD2 levels in healthy, or particularly at-risk women, could advance our ability to provide ovarian cancer prevention or risk reduction options, by trafficking FANCD2 into the nucleus.

## **Methods**

### **Patients and Specimens**

All patients underwent ovarian cancer staging and debulking surgery when this was considered an effective option. Tumors were classified according to World Health Organization criteria. All samples and the medical records of patients were retrospectively reviewed under an approved Institutional protocol that required written patient consent. The review included out- and in-patient treatment, which included surgery and chemotherapy. All patients undergoing chemotherapy received platinum-based treatments as a first line option. Overall survival and progression times were determined, each measured from the time of diagnosis at initial surgery. Progression was defined as objective evidence of recurrence by imaging studies. The duration of overall survival was the interval between diagnosis and death. Observation time was the interval between diagnosis and last contact (death or last follow-up). Data were

censored at the last follow-up for patients with no evidence of recurrence, progression or death.

### **Tissue Microarray Analysis:**

Tissue Microarray Analysis (TMA) was performed to detect FANCD2 expression and subcellular localization in 181 paraffin-embedded samples essentially as described (Kononen et al., 1998; Wysham et al., 2012). Briefly, 0.6 mm cores were drawn from each block (donor blocks) and transferred to microarray blocks (receiver blocks). To overcome tumor heterogeneity, core samples were taken from three different areas of each tumor. Receiver blocks were sectioned, and individual sections were labeled with H&E, to identify the presence of tumor, or probed with an antibody to FANCD2 (Epitomics, San Francisco, CA), which was visualized with a peroxidase/diaminobenzidine chromatic reaction (Envision Detection System, DAKO). As a negative control, receiver block sections were also labeled without exposure to primary antibody.

Subcellular localization and expression levels were determined for both cytoplasmic and nuclear fractions, by grading the staining intensity on a scale of 0-3, representing a range of background to strong signal. The grading was performed manually by an experienced Gynecologic Oncology pathologist. The extent of immunochemical reactivity was graded based on intensity as follows: 0 (background), 1 (light), 2 (moderate), 3 (strong).

### **Cell Culture, Fractionation and Western Blot:**

Cells were obtained from OOCRTR-banked, liquid nitrogen-frozen samples representing normal ovarian cells and ovarian tumors. Cultures were grown in defined medium: 50/50 DMEM/RPMI 1640, supplemented with EGF (0.01 µg/ml), Gentamycin (50 µg/ml), Cipro (10 µg/ml), Insulin (10 µg/ml) and Penicillin/Streptomycin (100 µg/ml). Cells were grown to approximately 80% confluence prior to harvesting.

Nuclear and cytoplasmic fractions were isolated using a Nuclear Extract Kit (Active Motif, Carlsbad, CA, version C4) per manufacturer's instructions. This separates the fractions by hypotonic lysis of the cytoplasm, to isolate cytoplasmic proteins, followed by complete lysis of nuclei via detergent and denaturation of each fraction.

Purified fractions were assayed for protein concentration using a Bradford-based method (BioRad) and prepared for electrophoretic separation of proteins in both nuclear and cytoplasmic fractions. Samples were denatured in reducing buffer, heated to 95°C for 10 minutes, and run on a 3-8% Tris-Acetate gel (Invitrogen; Carlsbad, CA). Following SDS/PAGE, proteins were transferred to nitrocellulose membranes, which were probed for FANCD2 (1:100) or nucleoporin (1:200) (Santa Cruz Biotechnology; Santa Cruz, CA), as an additional loading control. HRP-conjugated secondary antibodies were then used for signal detection. Bands were visualized using Supersignal West Pico and West Femto chemiluminescence (ThermoScientific; Rockford IL) on Blue Basic Autorad film (BioExpress; Kaysville, UT).

The level of expression of FANCD2 fractions in normal versus cancer samples was quantified relative to the expression in a control cell line transfected with SV40, which acts as a laboratory standard. Densitometric analysis of band intensity was performed using NIH ImageJ software.

## Statistical Analysis

Correlation between clinical and pathologic parameters, in the context of FANCD2 levels and subcellular localization was performed by analyzing a total of 181 patient samples. Survival analysis was conducted to determine survival difference between positive versus negative cFANCD2 using the Kaplan-Meier method, followed by Log-rank test. Significance of expression of FANCD2 between normal ovarian cells and ovarian cancer cells *in vitro*, was determined using Student's t-test of densitometric data described above. Statistical significance was defined as a p-value of  $<0.05$ . All the statistical analyses were performed using SAS statistical software (version 9.4: SAS Institute Inc, Cary, NC) or Microsoft Excel (version 12.3.1).

## Results

The presence of cFANCD2 in TMA samples significantly ( $p < 0.04$ ) associated with an increased survival with median survival times of 50 months in the positive cFANCD2 patients, versus 38 months in the negative cFANCD2 patients (Figure 1). We compared FANCD2 expression *in vitro* using ovarian cells from normal patients ( $n=3$ ) or ovarian cancer patients ( $n=4$ ). Western Blot and densitometric analysis that normalized band density to a control, transfected cell line (N/SV40) revealed significantly higher levels of nuclear FANCD2 in normal; i.e. primary, cells ( $0.858 \pm 0.254$ ) versus cancer cells ( $0.229 \pm 0.176$ ), with  $p < 0.02$ . Likewise, cFANCD2 was higher in normal ( $0.213 \pm 0.036$ ) versus cancer cells ( $0.107 \pm 0.044$ ), with  $p < 0.02$  (Figure 2). When the ratio of nuclear versus cFANCD2 levels were quantified against the normalized control (N/SV40), the



results indicated a trend difference ( $p = 0.09$ ), with a higher ratio of nuclear versus cFANCD2 in normal ( $4.20 \pm 2.00$ ) versus cancer ( $1.91 \pm 0.98$ ) cells.

## **Discussion**

In the present study, we investigated the prevalence of cFANCD2 in 181 ovarian tumor samples. Tissue Microarray Analysis revealed appreciable levels of cFANCD2 among these samples and, more importantly, a correlation between cFANCD2 expression levels and patient survival. Patients with higher levels of cFANCD2 had significantly ( $p < 0.04$ ) longer median survival (50 months), as compared with patients expressing lower cFANCD2 (38 months).

Levels of FANCD2 expression in general, and levels of cFANCD2 expression in particular, showed a consistent and significant ( $p < 0.02$ ) reduction in ovarian cancer cell lines versus normal ovarian cell lines that were cultured from samples banked in the OHSU OOCRTR. Although the current methodology was not applied to a direct measure of the levels of cFANCD2 versus nuclear FANCD2 in normal versus cancer lines, a comparison of normalized Western Blot data showed a trend ( $p = 0.09$ ) between the ratio of nuclear versus cytoplasmic FANCD2 in normal ( $4.24 \pm 2.00$ , nuclear/cytoplasmic) versus cancer ( $1.93 \pm 0.98$ ) cells. This suggests that not only do normal ovarian cells express a higher level of FANCD2 than do cancer cells, but that normal cells also contain a larger relative pool of nuclear FANCD2 compared with the cytoplasmic pool. It may be inferred, though not yet concluded, that a tangible cellular defect predisposing normal cells to transformation may be due to inappropriate subcellular localization of FANCD2 to the cytoplasm, where it would be unable to act in

conventional means at the site of DNA damage within the nucleus. While this may be important in disease occurrence, loss of cFANCD2 is also an indicator of poorer prognostic outcome once the disease has arisen (Rudland et al., 2010). Currently this is a complex scenario in which the overall levels of FANCD2 are relevant to ovarian cancer risk (Pejovic et al., 2006), while a subsequent preferential decrease in nuclear FANCD2 versus cFANCD2 results in susceptibility to platinum-based therapies; i.e., increased compartmentalization may enhance the efficacy of platinum-based therapies.

The trafficking of FANCD2 to the cytoplasm, or its retention therein, may not be a defect *per se*. Instead, there is evidence that components of the FA/BRCA pathway, including FANCD2 have noncanonical functions in the cytoplasm of normal cells (D'Andrea, 2003), and possibly cancer cells (Rudland et al., 2010). In this context, recent work using breast cancer samples demonstrated a loss of nuclear FANCD2 in benign and malignant tumors, with retention of cFANCD2, except in cases of malignant, highly invasive tumors (Rudland et al., 2010). This suggests a cytoplasmic function of cFANCD2 that limits invasiveness. To date, however, a mechanistic explanation of this cellular activity by cFANCD2 has not been delineated.

The observation that alterations in FANCD2 expression are associated with cancer prognosis is not novel (Wysham et al., 2012; Mhawech-Fauceglia et al., 2014); however, an analysis of subcellular localization of FANCD2 to the cytoplasm along with a correlation with ovarian cancer patient survival has not been previously reported. Previous studies have demonstrated profound influences of FA/BRCA defects in a number of cancers, including ovarian cancer (D'Andrea, 2003; Lyakhovich and Surralles, 2006; Garcia and Benitez, 2008). Indeed, previous work from this laboratory

demonstrated that a high proportion of women with a family history of breast and/or ovarian cancer exhibited defects in FANCD2 expression: in that study (Pejovic et al., 2006), 60% of ovarian cells obtained from women with a family history of the disease displayed low or absent levels of FANCD2. In that study, no distinction was made between nuclear and cytoplasmic compartmentalization of FANCD2; however, in light of current findings, this warrants further investigation.

Exploring the molecular basis of cancer has historically focused on genetic aberrations that manifest as mutations, or deletion or amplification of genes. As our understanding has evolved, the mechanisms of tumorigenesis can be seen as manifestly more complex. Research into the epigenetic regulation of tumor promoters or suppressers and/or alterations in protein trafficking of, for example, components of the FA/BRCA DNA repair pathway (Pejovic et al., 2006; Taniguchi and D'Andrea, 2006; Henderson, 2012; Seeber and van Diest, 2012) have been more difficult to detect in epidemiological approaches or via genetic screening to detect cancer-associated gene defects. However, identifying and understanding the array of molecular mechanisms associated with diverse tumor types, even those originating in identical tissues, may improve the availability of patient-specific, tailored therapies for detection, prevention and treatment. Noncanonical aspects relating to cancer cell biology include alternative splicing, post-translational modifications and intracellular trafficking.

We hope to elucidate the mechanism of FANCD2 compartmentalization. Due to the presence of a nuclear localization signal at the carboxy terminus of FANCD2 it will be important to distinguish between a failure of a fraction of the total pool to be transported into the nucleus and/or mechanisms by which the nuclear pool is depleted, even in the

presence of DNA damage that normally results in FANCD2 activation (Kim and D'Andrea, 2012). These could include post-translational modifications, independent of ubiquitination, or the association of cFANCD2 with cytoplasmic proteins that prevent nuclear import. Additional studies should aim to identify cytoplasmic roles for FANCD2 that promote or suppress tumorigenesis or influence tumor invasiveness.

The significance of functional defects in FANCD2, absent genetic mutation, in ovarian cancer risk and patient survival has been established here and in prior studies (Pejovic et al., 2006; Wysham et al., 2012) and includes overall expression levels and, in this novel study, cytoplasmic expression. Therefore, further investigation of FANCD2 regulation may yield significant clinical benefit.

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**Legends:**

**Figure 1.** Survival time after initial diagnosis, segregated on the basis of positive versus negative cFANCD2. Red line records positive cFANCD2 samples; with a median patient survival of 50 months. Blue line records patients that were absent for cFANCD2; with a median survival time of 38 months. Overall survival was significantly higher ( $p < 0.05$  by Kaplan-Meier and Log-Rank test) in patients with lower cFANCD2.

**Figure 2.** Western Blot shows nuclear (top panel) and cytoplasmic (bottom panel) localization of FANCD2 in normal (N1-3) OSE versus ovarian cancer cells (CA1-4). N/SV40 is used as an internal control. 10  $\mu$ g of protein from each sample was loaded per lane. Densitometric analysis demonstrates significant difference between normal versus cancer cells in both the nuclear fraction ( $p < 0.02$ ) and cytoplasmic fraction ( $p < 0.02$ ).



# Estradiol does not modulate FANCD2 in high risk ovarian surface epithelial cells

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## Background

Approximately 10-15% of ovarian carcinomas, thought to arise from the ovarian surface epithelium (OSE), occur due to inherited mutations in *BRCA1* and *BRCA2*, which confer a 20-50% lifetime risk.<sup>1</sup> However, *BRCA1* and *BRCA2* mutations account for only a fraction of inherited ovarian cancers. Consequently, other alterations must account for the remaining risk. We have found that the majority of high-risk women (without *BRCA* mutations) display low levels of the protein FANCD2. FANCD2 is part of a large complex of proteins, including *BRCA1* and *BRCA2*, that mediates chromosomal stability and is collectively referred to as the Fanconi anemia FA/BRCA pathway. Eight of the FA genes form a nuclear complex that facilitates the monoubiquitination of the downstream FANCD2 protein. The monoubiquitinated form can be distinguished by western blot.

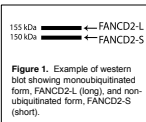


Figure 1. Example of western blot showing monoubiquitinated form, FANCD2-L (long), and non-ubiquitinated form, FANCD2-S (short).

This monoubiquitination allows the FANCD2 protein to co-localize with *BRCA1*, *BRCA2*, and *RAD51* in damage-induced nuclear foci.<sup>2</sup>

## Background cont.

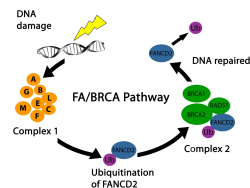


Figure 2. DNA damage occurs and Complex 1 monoubiquitinates FANCD2 in the nucleus. *BRCA1/2* and *RAD51* combine with FANCD2-Ub to co-localize in damage-induced nuclear foci. The DNA is repaired and enzyme *USP1* deubiquitinates FANCD2-Ub to complete the pathway.

Several lines of additional evidence support an essential role of the FA/BRCA pathway in suppressing ovarian cancer *in vivo*, including spontaneous ovarian tumor formation in *fancd2*-deficient mice and high levels of epigenetic silencing of *FA/BRCA* genes in sporadic ovarian cancers (including *FANCF*).<sup>3</sup> We and others have shown that alterations in FA genes account for a proportion of non-*BRCA* hereditary ovarian cancers.

The preponderance of tumors in sex hormone-responsive tissues (specifically breast and ovary) in *FA/BRCA* carriers and the high levels of acquired *FA/BRCA* alterations in these same tissues in sporadic breast and ovarian cancers suggest that estrogen may play an essential role in development of these cancers. 17 $\beta$ -estradiol, the most biologically active estrogen *in vivo*, induces chromosomal aberrations and other types of damage *in vitro* and *in vivo*.<sup>4</sup>

## Background cont.

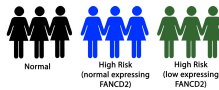
Because FA pathway members (including FANCD2) mediate chromosomal stability in response to genotoxic agents and a significant proportion of ovarian cancers have alterations in *FA/BRCA* genes (inherited or acquired), we hypothesize that estrogen functions *in vivo* to promote ovarian tumor development by inducing chromosomal instability in OSE cells.

## Methods

Patients at high risk for ovarian cancer were defined as women with:

- 1<sup>st</sup> degree relative with ovarian cancer before age 50
- 1<sup>st</sup> degree relative with ovarian cancer diagnosed at any age and one or more 1<sup>st</sup> or 2<sup>nd</sup> degree relative with breast or ovarian cancer
- Personal history of breast cancer and one or more 1<sup>st</sup> or 2<sup>nd</sup> degree relative with breast or ovarian cancer

Cells from patients undergoing primary surgery for benign gynecologic disease with no family history of ovarian cancer were also collected and cultured and defined as normal population (n=3). Cultured cells were screened for expression of FANCD2 by immunoblot and were segregated on the basis of expressing FANCD2 at low levels (n=3) or normal levels (n=3).



Cells were cultured in the presence of 17 $\beta$ -estradiol at 3  $\mu$ M for 72 hours and were harvested to separate nuclear and cytoplasmic compartments. Separation of these compartments was performed using hypotonic-based isolation of cytoplasmic components from nuclei, each compartment was lysed and immunoblot was performed.

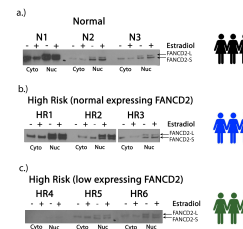
## Methods cont.

### Immunoblot

- 10  $\mu$ g of total protein from each fraction
- Protein separation by SDS/PAGE and transferred to nitrocellulose
- Probe for FANCD2
- Loading controls: alpha-tubulin for cytoplasmic fraction; nucleoporin p62 for nuclear fraction.
- NL9/sv40 cell line used to establish baseline of FANCD2 expression

### Results

Estradiol treatment had no detectable effect on FANCD2 expression in any of these cell lines in either the nuclear or cytoplasmic compartments.



FANCD2 was detected in both cytoplasmic and nuclear fractions of normal lines and normal expressing FANCD2 lines from women at risk for ovarian cancer. FANCD2 was low in both fractions in lines from women at risk but expressing low FANCD2. The ubiquitinated form of FANCD2 (FANCD2-L) was only detected in the nuclear fraction.

## Conclusions

Estradiol had no discernible effect on FANCD2 expression, regardless of the risk-status of the cell line. The absence of response was seen in nuclear and cytoplasmic fractions. The use of cells expressing normal or low FANCD2 allowed the potential to observe effects of estradiol on either increasing or decreasing FANCD2; however, neither effect was detected. Additional studies remain to be conducted using estrogen metabolites, and to examine the degree of FANCD2 activation (ubiquitination) of the nuclear fraction of FANCD2.

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